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Role of drug-induced Fas ligand expression in breast tumor progression
Annual report

(4) Introduction

Fas-induced apoptosis is a well-defined apoptotic pathway (1, 2). Interaction of Fas/FasL leads to recruitment of death-associated proteins to the cytoplasmic domain of Fas. Depending on which adaptor protein associates with Fas, two distinct Fas-mediated pathways have been identified, i.e., caspase-dependent and caspase-independent pathway. FADD mediates a caspase-dependent pathway through recruitment of pro-caspase-8, whereas Daxx mediates a caspase-independent pathway by activating a pro-apoptotic kinase, apoptosis signal-regulating kinase 1 (Ask1) (3, 4).

Although the Daxx pathway is not as well-known as the FADD pathway, the role for Daxx in Fas-induced apoptosis is relatively well defined. While the FADD pathway plays a major role at the early stage, the Daxx pathway is believed to play a role in a late stage (5). Daxx was first identified as a protein that binds to the cytosolic domain of Fas and links this receptor to an apoptosis pathway involving activation of Jun N-terminal kinase (JNK) (3). Daxx resides in both the cytoplasm and nucleus; and it has a dual function. While the cytosolic Daxx participates in Fas-induced apoptosis, the nuclear Daxx acts as a negative transcriptional regulator (6). In the cytoplasm, Daxx interacts with death domain of Fas and Ask1, and activation of Ask1 by binding Daxx leads to activation of JNK. On the other hand, the nuclear Daxx controls expression of several important proteins such as PAX3, which is a transcription factor that plays an important role in development. Deregulated expression of Pax3 has been implicated in tumor cell survival (7, 8).

Consequently, regulation of Daxx for its role in enhancing Fas-mediated apoptosis seems to be complex and several proteins have been shown to be involved in this process. For instance, Ask1 appears to play an important role in controlling the subcellular localization of Daxx because overexpression of Ask1 causes translocation of Daxx from the nucleus to the cytoplasm and formation of Fas/Daxx/Ask1 complexes (9). By contrast, HSP27 has been shown to participate regulatory network of the cytoplasmic Daxx by blocking Fas-induced translocation of Daxx from the nucleus to the cytoplasm and Fas-induced Daxx- and Ask1-dependent apoptosis. Phosphorylated dimers of HSP27 interact with Daxx, preventing the interaction of Daxx with both Ask1 and Fas and thus, blocking Daxx-mediated apoptosis (5). Therefore, subcellular distribution of Daxx is a key for the function Daxx plays in the cell. However, whether interaction of Daxx with UBC9 facilitates the nuclear import of Daxx is not known, nor the effect of anticancer drugs on the subcellular localization of Daxx.

All anticancer agents seem to be able to kill tumor cells through programmed cell death (PCD), although their primary targets may be different. It has been previously shown that FasL is induced by anticancer agents as well as other DNA damaging agents (10). However, the role of FasL induction in cytotoxicity of the drug has been controversial presumably because different cell lines seem to have different consequences. Although a number of groups reported FasL induction by anticancer drugs including topoisomerase (topo) inhibitors in vitro, whether this FasL induction also occurs in vivo is not known. In the past year, we have been trying to address this issue by using a mouse tumor model. Our results, to be described below, indicate that treatment of

animals with VP-16 or doxorubicin produced no significant induction of FasL in our mouse tumor model. However, we found that Daxx is induced by topo II inhibitors, and it interacts with UBC9, an E2 conjugating enzyme that is required for sumoylation (11). More importantly, we found that the drug treatments decrease the binding activity of Daxx to UBC9, suggesting that the drug may have an impact on the subcellular redistribution of Daxx. Given the fact that the subcellular localization of Daxx is important for its function, we believe that the impact of topo inhibitors on Daxx and its interaction with UBC9 could be attributable to the cytotoxicity of the drugs through regulation of down-stream components of the Fas-mediated apoptosis pathway.

(5) Body

FasL expression in mouse mammary tumor cells after treatment with topo II inhibitors

As described in the previous annual report, we found no induction of FasL by VP-16 in the human breast cancer cell line MCF-7 in SCID mice, but we found by RT-PCR some FasL induction by this drug treatment in the mouse mammary tumor cell line CRL-2116 in wild type mice. Therefore, we focused on CRL-2116 cells in the past year. We have tested a total of 128 mice. In pilot experiments, we found that, in general, tumors grew relatively fast and lasted for about 4 weeks before tumors were harvested. To test the toxicity of VP-16 to mice, we also carried out some pilot experiments. Thus, we treated mice with different doses of VP-16 (10, 15, 20, 25 and 30 $\mu\text{g/g}$ body weight, respectively) one week after the inoculation with CRL-2116 tumor cells. The maximum tolerated dose (MTD) for the tumor-bearing mice is 15 $\mu\text{g/g}$ body weight. Although we used near MTD of VP-16, the tumors grew slightly more slowly than those with vehicle alone. A representative tumor growth curve from a pool of 10 mice is shown in Fig. 1A.

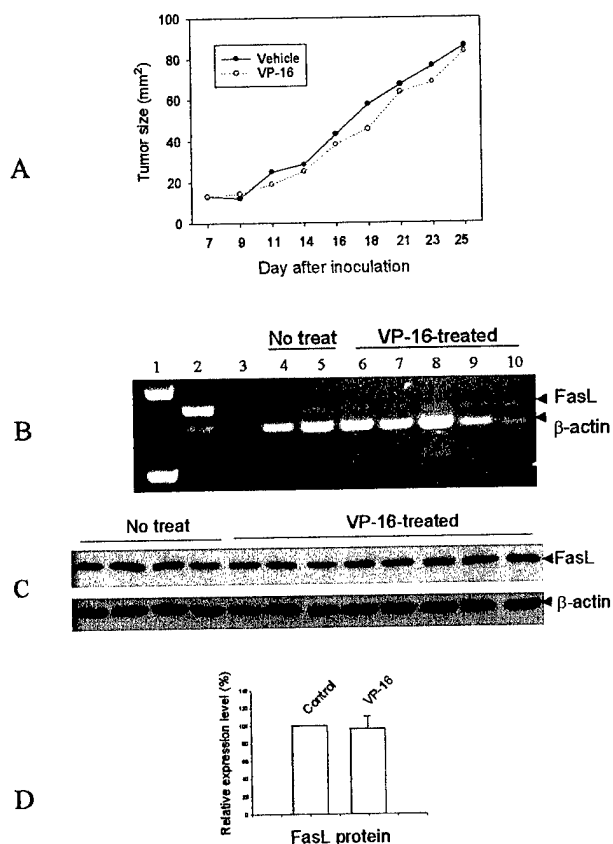


Fig. 1 Effect of VP-16 on FasL expression in tumors. **A**, A tumor growth curve from 10 mice. Tumor-bearing mice were treated with 12.5 μg VP-16/g body weight. **B**, Expression of FasL mRNA as detected by RT. Shown here is a representative picture of some tumor samples. Lane 1, DNA marker; lane 2, spleen serving as a positive control; lane 3, a negative control. β -actin serves as a loading control. **C**, A representative expression of Fas protein as detected by Western blot. **D**, Overall expression of FasL protein (Western blot data) from a total 25 mice. There is no significant difference between control and VP-16 (Student Test, p value = 0.51).

Next, we examined FasL expression from these tumors. The tumors were removed and frozen in dry ice immediately. Total RNA and protein extract were prepared separately. As shown in Fig. 1B and C, we detected no significant induction of FasL by either RT-PCR or Western Blot. The RT-PCR results seemed to be more various than the Western blot data, i.e. we observed in some tumor samples about 1.5-2 fold increase in

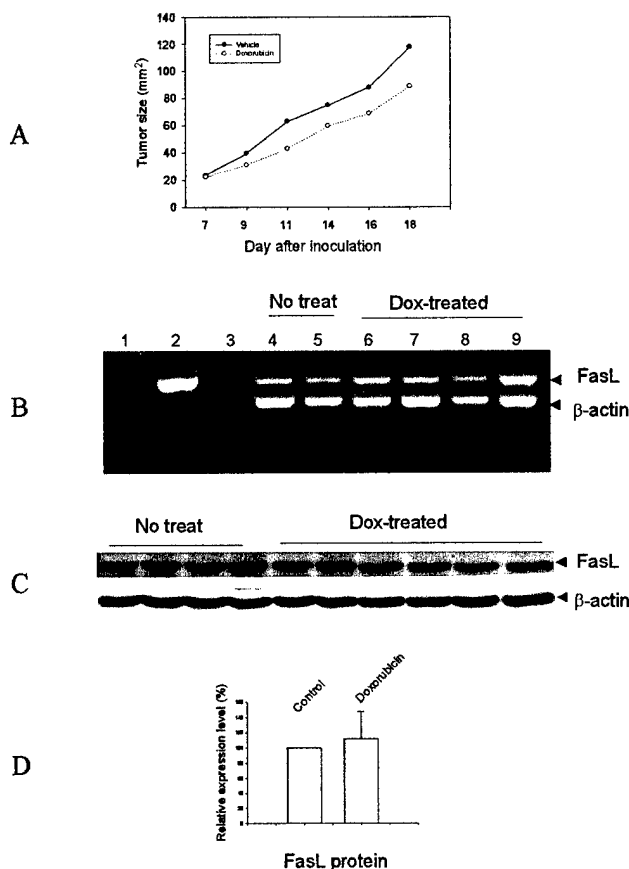


Fig. 2 Effect of doxorubicin on FasL expression in tumors. **A**, A tumor growth curve from 10 mice. Tumor-bearing mice were treated with 2 μ g doxorubicin/g body weight. **B**, A representative picture of some tumor samples for mRNA levels as detected by RT-PCR. Lane 1, DNA marker; lane 2, spleen serving as a positive control; lane 3, a negative control. **C**, A representative expression of Fas protein as detected by Western blot. **D**, Overall expression of FasL protein (Western blot data) from a total 20 mice. There is no significant difference between control and doxorubicin (Student Test, p value = 0.49).

expressing this construct, we detected its expression under fluorescence microscope. Therefore, we introduced this construct into CRL-2116 cells. In vitro tests revealed some induction after treatment with doxorubicin or VP-16 (Fig. 3B). However, when the same cells were inoculated onto the mice, the green fluorescent signal in tumors from those mice was too weak to be detected.

FasL expression as compared with the control, but in most cases, like Western blot data, no obvious induction was seen. Overall, there was no statistically significant difference between control and treatment, with P value of 0.51 (Fig. 1D).

Since there was no significant induction of FasL from VP-16-treated tumors, we then tested another topo II inhibitor, doxorubicin (Dox), which served as an alternative drug. Again we first tested MTD for the tumor-bearing mice. Four doses of doxorubicin (1, 2, 3 and 4 μ g/g body weight, respectively) were tested. The MTD was 2 μ g/g body weight. Thus, we used 2 μ g/g body weight for the following experiments (Fig. 2A). As for VP-16 treatment, RT-PCR and Western blot were performed to monitor the FasL expression. A representative RT-PCR and Western blot is shown in Fig. 2B and C. Similar to the data for VP-16, there was no significant induction of FasL (Fig. 2D).

To try an alternative approach to monitor FasL expression, we used FasL promoter-EGFP. To increase the detection sensitivity, we introduced two copies of FasL promoter (Fig. 3A). As we previously showed in HeLa cells

In summary, we have repeated these experiments many times by different approaches (i.e., different drugs and different detection methods). Although there might be a low level of FasL induction in some tumors, overall there is no significant FasL induction. We believe that the tumor cells do not respond well to these drugs because

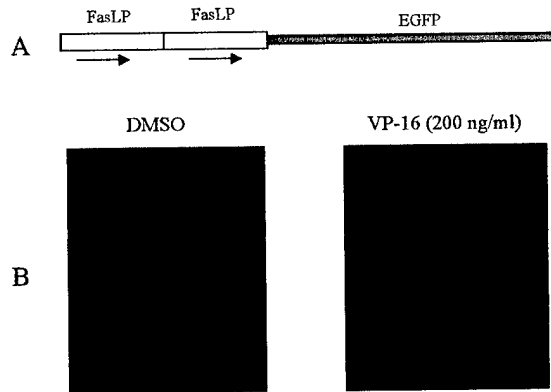


Fig. 3 Measurement of FasL expression in CRL-2116 cells in vitro by the FasL-EGFP reporter. **A**, A construct of FasL-EGFP used in this study. Two copies of FasL promoter were fused with the coding region of EGFP (pEGFP-1, Clontech). **B**, Cells of a stably transfected CRL-2116/FasL-EGFP were treated with DMSO or VP-16 at 200 ng/ml for 48 h and examined under a fluorescence microscope (Carl Zeiss) for the same exposure time. A slight increase in the intensity of fluorescence was seen in treated cells.

we did not see a good inhibition of tumor growth even though we used maximum tolerated doses of drugs. Furthermore, the high toxicity of the drugs to the mice seems to be important. Unlike the in vitro experiments, tumor-bearing mice are very sensitive to the toxicity of drugs, so that the drug concentration tested has to be kept low. Another possibility is that the exposure time to the drug is relatively short due to the severe effect of tumors on the mice. These factors might contribute to the difficulty in detecting FasL induction in vivo. Therefore, we plan to test other anticancer agents such as cis-platin, cytoxan and 5-fluorouracil in the coming year in the hope of seeing a tumor response. If we see good tumor growth inhibition or especially cell killing, we will examine the tumors for induction of FasL.

Daxx

In the process of doing these experiments, however, we made substantial progress regarding Daxx, as summarized herein. Daxx has been shown to interact directly with Fas and implicated in the enhancement of Fas-induced apoptosis. Particularly, our results suggest that UBC9 may play a role in regulating the subcellular localization of Daxx, and drug treatment may affect this regulatory network, thus enhancing apoptosis.

Daxx physically interacts with UBC9 and is induced by topo II inhibitors

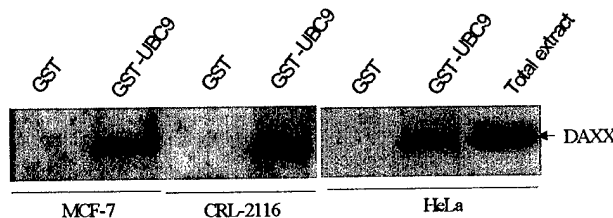


Fig. 4 Interaction of Daxx with UBC9 by GST pull-down assays. Log phase cells (HeLa, CRL-2116 and MCF-7) were harvested and lysed in the GST pull-down buffer. After centrifugation, the supernatants were collected and mixed with GST-UBC9 beads, followed by incubation for 2 h at 4°C. Then, the beads were washed for three times and resuspended in the protein sample buffer. The membrane was probed with anti-Daxx antibody.

As an alternative pathway leading to cell death, Daxx plays an important role in enhancing Fas-mediated apoptosis at the later stage (5). More importantly, the function of Daxx depends on its subcellular localization; only the cytosolic Daxx is involved in Fas-induced apoptosis process. However, the subcellular redistribution of Daxx in response to different stimuli is poorly understood. Protein modification is thought to be

important, and small ubiquitin like modifiers (SUMO) appear to play a role in some aspects of this phenomenon. Sumoylation of proteins requires UBC9, an E2 conjugating enzyme. Therefore, we asked whether UBC9 binds to Daxx. Using HeLa cell extracts and GST pull-down assays, we detected a ~120 kDa protein band that was recognized by Daxx-specific antibody. This band was specific to UBC9 because no such a band was detected in the "GST alone" lane. Then we tested cellular extract from mouse mammary tumor cells (CRL-2116) and human breast cancer cells (MCF-7). As with the HeLa extract, only UBC9-GST pulled down Daxx from the extracts of these two cell lines (Fig. 4), suggesting that Daxx specifically interacts with UBC9.

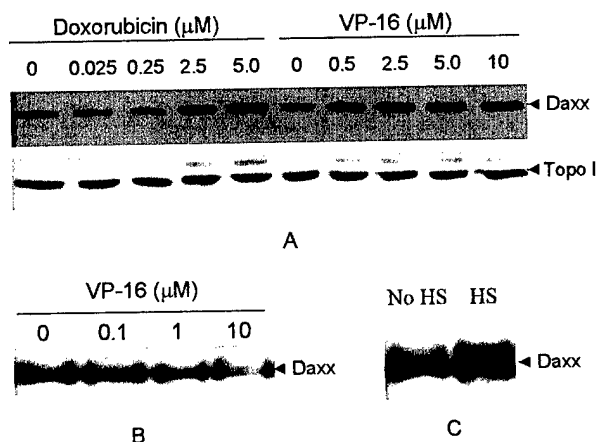


Fig. 5 Effect of topoisomerase inhibitors and heat shock on expression of Daxx and the binding of Daxx to UBC9. **A**, Induction of Daxx in CRL-2116 cells by VP-16 and doxorubicin. Cells were treated with the drugs for 8 h at the concentrations as indicated. Topo I was used as a loading control because the level of topo I was found not to be affected by topo II inhibitors. **B**, Reduction of Daxx binding to UBC9 by VP-16. CRL-2116 cells were treated with VP-16 for 24 h at the concentrations as indicated. The same amount of total protein (~250 μg) for each sample was used for UBC9-GST pull-down assays. **C**, Enhancement of Daxx binding to UBC9 by heat shock. MCF-7 cells were subjected to heat shock (43.5°C for 30 min) and lysed immediately. About 250 μg protein was used for GST pull-down assays.

treatment, the heat shock enhanced the binding activity of Daxx to UBC9 (Fig. 5C), while the amount of Daxx protein used for the binding assays was about the same. These results suggest that drug treatment may keep more Daxx in the cytoplasm while the heat shock shifts the balance toward the nucleus.

Drug effect on subcellular redistribution of topo I

To test a possible role of UBC9 and sumoylation in subcellular localization of targeted proteins, we have examined a relationship between sumoylation of topo I and its nucleolar delocalization. Our results indicate that sumoylation of topo I might serve as an addressing tag because there is a good correlation between sumoylation and its nucleolar delocalization (Fig. 6). After exposure to topotecan, we detected multiple bands that

Next, we tested the effect of topo inhibitors on expression of Daxx and its interaction with UBC9. We treated CRL-2116 cells with VP-16 for 8 h and cellular extract was prepared. We found that VP-16 induced expression of Daxx (Fig. 5A). Similarly, the level of Daxx protein increased after treatment with doxorubicin. However, the same drug (VP-16) tended to decrease the binding activity of Daxx to UBC9 (Fig. 5B), suggesting that the

drug may cause some modification of Daxx as well as other proteins so that the binding activity of Daxx to UBC9 is altered.

Since HSP27 has been shown to block the binding of Daxx to Fas and Ask1 (5), we examined the effect of heat shock on the binding of Daxx to UBC9. Thus, we treated MCF-7 cells for 30 min at 43.5°C. As a control, another dish of cell culture was kept for 30 min at 37°C. In contrast to drug

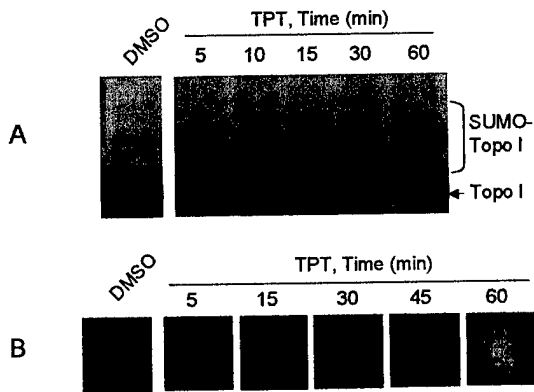


Fig. 6 Comparison of temporal changes for sumoylation and subnuclear localization of EGFP-topo I fusion protein. **A**, Sumoylation of topo I at different time points. HeLa cells were treated with 10 μM TPT for various times as indicated and blotted with topo I specific antibody. The same multiple bands were also recognized by anti-SUMO antibody (not shown). **B**, Temporal changes for subnuclear localization of EGFP-topo I fusion protein. The transfected HeLa cells were exposed to 10 μM TPT for times indicated. Note that sumoylation of topo I occurs before the nucleolar delocalization is seen.

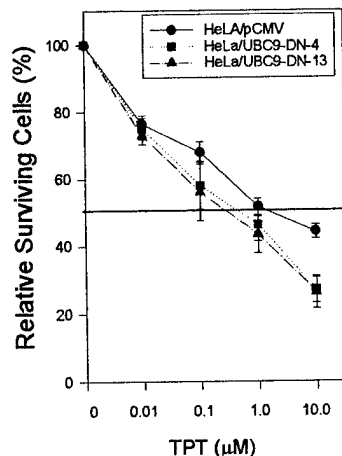


Fig. 7 Effect of UBC9-DN on the sensitivity of HeLa cells to the topo I inhibitor topotecan as measured by MTT assays. Cells were seeded in a 96-well plate and grown overnight. Drug was added at the concentrations as indicated and incubated for 48 hr before MTT assays. The values are average \pm SD of three separate experiments

drug resistant sublines CEM/VM-1 and CEM/VM1-5 are 40 and 140 fold more resistant

reacted with both topo I and SUMO antibodies (Fig. 6A); at the same time, nucleolar EGFP-topo I fusion protein was disappearing as the exposure time increases (Fig. 6B). Furthermore, UBC9 seems to play an important role in nuclear delocalization of topo I because overexpression of a dominant negative UBC9 inhibited this nucleolar delocalization (not shown).

Deregulation of UBC9 is associated with the resistance of tumor cells to anticancer drugs

In addition to its requirement for sumoylation, UBC9 has also been implicated in DNA repair because UBC9 physically interacts with Rad51 (12). We sought to determine whether alteration of UBC9 expression has any effect on drug responsiveness. A point mutation (C93S) in UBC9 has been shown to have a dominant negative effect on the endogenous UBC9. Thus, we introduced

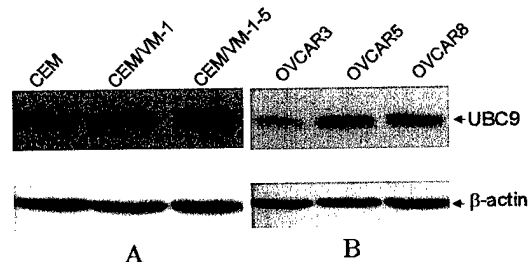


Fig. 8 Association of deregulated expression of UBC9 in tumor cell lines with drug resistance. Total protein was prepared from log phase cells. The membranes were probed with anti-UBC9 antibody. β-actin was used as a loading control.

this dominant negative mutant into HeLa cells. When overexpressed, the dominant negative mutant UBC9 cells become more sensitive than the vector control (Fig. 7), suggesting that UBC9 plays a role in drug responsiveness. Next we screened some existing resistant cell lines in this laboratory for expression of UBC9. CEM is a T-cell lymphoblastic parental cell line that is sensitive to VM-26, a complex stabilizing topo II inhibitor, whereas its

to VM-26, respectively, than CEM cells. We consistently observed up-regulation of UBC9 in CEM/VM-1-5 cells (Fig. 8A); similarly, we also found that the expression level of UBC9 in CEM/VM-1 cells was higher than that of CEM cells, although at a less extent.

We also examined three ovarian cancer cell lines that expressed different levels of resistance to cis-platin, a DNA cross-linking agent. There seemed to be a good correlation between resistance to cis-platin and expression level of UBC9. For instance, IC₅₀ for OVCAR3, OVCAR5 and OVCAR8 are 2.7, 5.3 and 10 μ M of cis-platin, respectively. We detected higher levels of UBC9 protein in OVCAR5 and OVCAR8 than in the most sensitive cell line OVCAR3 (Fig. 8B). Semi-quantitative RT-PCR revealed similar results to those of Western blot (not shown).

Future work for next year

While we will continue our effort to search for more potent but less toxic drugs and then test their effect on FasL expression, we would like to devote part of our effort to further investigation on the interaction between Daxx and UBC9 in the context of the subcellular localization and drug responsiveness. Daxx exists in both the cytoplasm and the nucleus. In the cytoplasm, Daxx mediates the apoptotic signal from Fas to JNK; on the other hand, the nuclear Daxx functions as a transcriptional regulator. Thus, the subcellular localization of Daxx is critical for its action. It has been recently shown that the relative concentration of a pro-apoptotic kinase, Ask1, controls the destination of Daxx because Ask1 sequesters Daxx in the cytoplasm and inhibits its repressive activity in transcription (13). In addition to Ask1, UBC9 may play an important role in regulating the nuclear transport of Daxx, because sumoylation has been implicated in subcellular localization of the targeted proteins (14) and UBC9, as an E2 enzyme, is required for sumoylation. For instance, through its SUMO-conjugating activity, Ubc9 is known to mediate the nuclear localization of the Rel family protein (15). In addition, several important proteins have been shown to be sumoylated, followed by subcellular redistribution, in response to environmental stimuli such as stress and drugs. For example, PML has been shown to regulate transcriptional activity of p53 through sumoylation/UBC9 (16-18). Thus, UBC9 is a very important player in this regulatory network. Our results (above) suggest that UBC9 may play a similar role in controlling the subcellular localization of Daxx. Topo inhibitors decrease the binding ability of Daxx to UBC9 (Fig. 5B), suggesting that the interaction of Daxx and UBC9 is an important step for the nuclear import of Daxx. In support of this notion, we have shown that topo I moves out of the nucleolus and translocates into nuclear body-like structures in response to topo inhibitors; overexpression of a dominant negative UBC9 tends to attenuate this process. Moreover, since UBC9 has also been shown to bind to RAD51 that has been implicated in DNA repair, UBC9 might play a role in drug responsiveness. In addition, several important nuclear proteins such as p53 have been shown to be sumoylated, leading to alteration of their activities and different physiological consequences (Fig. 9). Thus, it will be important to determine the effect of overexpression of the dominant negative UBC9 on drug sensitivity and even tumor growth potential. Specifically, we plan to investigate the effect of topo inhibitors on the subcellular localization of Daxx using EGFP-Daxx reporter constructs as well as its role in cytotoxicity of the drugs. We will also establish stable CRL-2116 or MCF-7 cells expressing the dominant negative

UBC9, and then test the drug responsiveness and tumorigenesis of these cells. We expect that these tasks will be completed within 12 months. Accordingly, we will modify our Statement of Work (also see a separate letter).

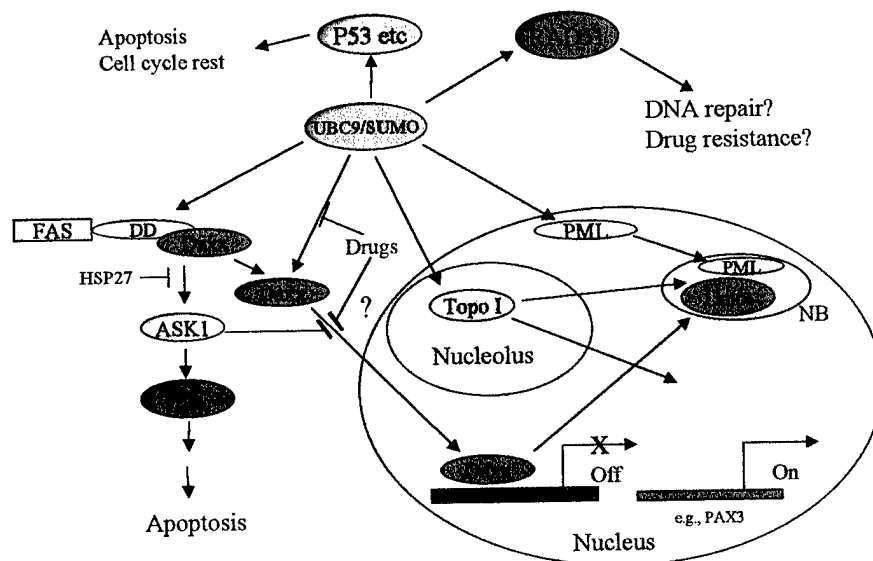


Fig. 9 A working model to test the role of Daxx and UBC9 in the drug responsiveness and apoptosis. This model is based on literature cited in this report as well as the results in this study. Although two Fas-induced apoptotic pathways, mediated by FADD or Daxx, have been identified, only the Daxx pathway is presented here. In order for Daxx to play a role in enhancing Fas-mediated apoptotic process, Daxx has to be in the cytoplasm. Activation of Fas leads to translocation of Daxx from the nucleus to the cytoplasm. Several other factors such as the amount of Ask1 have been shown to affect the subcellular distribution of Daxx. We would like to: 1) test whether topo inhibitors also affect its subcellular distribution, 2) determine the effect of UBC9 on the drug responsiveness and tumorigenesis by overexpressing UBC9-DN. Abbreviations: NB, nuclear body; DD, death domain; DN, dominant negative; SUMO, small ubiquitin-like modifiers; PML, promyelocytic leukemia protein; topo, topoisomerase.

Plan (Statement of Work)

Test other anticancer drugs such as cis-platin, cytoxan and 5-florouracil. Only those drugs that show a great reduction of tumor growth will be used for FasL induction experiments (months 1-9).

Examine the effect of drug treatment on subcellular localization of Daxx including construction of EGFP-Daxx plasmids (months 1-2).

Establish stable CRL-2116 cells or MCF-7 cells expressing dominant negative UBC9 and test the sensitivity to drugs in vitro (months 1-3).

Test the effect of dominant negative UBC9 on tumor growth and responsiveness of the tumors to drugs (months 3-12).

Data analysis, preparation of manuscripts and final report (months 9-12).

(6) Key research accomplishments

- ◆ Daxx is induced by anticancer drugs although no significant FasL induction was detected.
- ◆ Daxx physically interacts with UBC9, a protein that has been shown to be essential to sumoylation of many nuclear proteins; this interaction is adversely affected by topo inhibitors.
- ◆ Up-regulation of UBC9 is associated with increasing resistance to DNA damaging agents whereas overexpression of dominant negative UBC9 sensitizes the cell to these agents.

(7) Reportable outcomes

- ◆ Alteration of the interaction between Daxx and UBC9 by anticancer drugs
- ◆ A role for UBC9 in responsiveness of tumor cells to anticancer drugs

(8) Conclusions

During the last year, we have tested whether FasL is induced *in vivo* by the anticancer drugs VP-16 and doxorubicin using the mouse model. Although initial experiments with CRL-2116 cells seemed to support the notion the FasL is induced by VP-16 *in vivo*, we were not able to confirm these results with more experiments. However, while the results were negative, we found that Daxx, a Fas associated protein, is induced by drug treatment. Furthermore, GST pull-down assays revealed that Daxx binds to UBC9 and the binding activity of Daxx to UBC9 is adversely affected by topo inhibitors. Finally, we demonstrated that overexpression of UBC9-DN increases the sensitivity of tumor cells to topo inhibitors.

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